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Epidemiology of rabbit haemorrhagic disease virus in the United Kingdom: evidence for seasonal transmission by both virulent and avirulent modes of infection

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SUMMARY

Rabbit haemorrhagic disease virus (RHDV) has killed many millions of wild rabbits in Europe and Australia, but has had little impact in the United Kingdom, despite outbreaks having occurred since 1994. High seroprevalence detected in the absence of associated mortality had suggested the presence of an endemic non-pathogenic strain which may be 'protecting' UK populations. Following the first detailed field study of RHDV epidemiology in the United Kingdom, using mark–recapture with serum sampling, we report that RHDV caused highly prevalent persistent infection in seropositive rabbits in the absence of associated mortality. Furthermore the virus strains responsible could not be distinguished phylogenetically from known pathogenic isolates, and were clearly very different from the only previously identified non-pathogenic strain of RHDV. These findings suggest that many – perhaps most – strains of RHDV may be propagated through both 'pathogenic' and 'non-pathogenic' modes of behaviour. Transmission occurred predominantly during and just after the breeding season.

INTRODUCTION

Rabbit haemorrhagic disease virus (RHDV) is a highly virulent pathogen that kills up to 95% of infected rabbits within 48 h post-infection [1–3]. It has killed many millions of wild rabbits in Australia and Europe [3, 4]. A non-pathogenic strain of RHDV, which confers protective immunity to pathogenic RHDV, was isolated from an Italian rabbit farm in 1996, where it was highly prevalent and had persisted for at least 2 years [5–7]. Although this is the only case of a non-pathogenic strain having been identified and this strain has not been detected elsewhere, there have been numerous cases in which rabbits have been found to be seropositive to RHDV without any significant mortality attributable to the disease, including captive farmed and laboratory rabbits, in which the disease would certainly have been noticed [6, 8, 9]. Therefore it seems likely that the non-pathogenic strain(s) of RHDV may be widespread.

Non-pathogenic RHDV may be endemic in the United Kingdom, since a seroprevalence survey of 68 wild rabbit populations in the United Kingdom during January–March 1995 found that all populations had been infected with a mean seroprevalence of 64% (range 10–100%) [10–12]. None of these populations were known to have experienced rabbit haemorrhagic *disease* (RHD) – and since RHD has a very high case-fatality rate, and immune individuals were highly prevalent in most populations, mortality due to disease would have been apparent. This

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putative non-pathogenic strain appears to have protected many UK populations from pathogenic strains of RHDV, which have had little overall impact on the national population of rabbits – both in terms of the numbers of sites affected and the total number of rabbits killed – despite outbreaks having occurred country-wide since the first one in 1994 [13] (R. C. Trout & P. J. White, unpublished observations). Unfortunately, the seroprevalence survey was not repeated so there are no data regarding how seroprevalence changed over time.

This paper reports the first detailed study of the epidemiology of RHDV under field conditions in the United Kingdom. Three rabbit populations were regularly serum-sampled for up to 114 weeks by mark–recapture. Spreading of pathogenic and non-pathogenic RHDV was analysed at both the population level and the individual level, by detecting seroconversion of individuals and comparing mortality rates of seronegatives and seropositives. The aims of this study were (i) to determine whether a non-pathogenic strain of RHDV was endemic, and if so then when its transmission occurred; (ii) to quantify the impact of any outbreak of pathogenic RHDV; and (iii) to determine how long immunity to RHDV lasted.

METHODS

There were three study sites: Exminster (Devon, Ordnance Survey grid reference SX 942868); Frensham (Surrey, SU 847417); and Logiealmond (Perthshire, NN 910360). Rabbits were trapped using box, 'smeuse' and cage traps. The box traps (supplied by Lauderdale Engineering, Berwickshire, UK and by David Parker of Bentham, Lancaster, UK) consisted of a tunnel, inserted through a wire mesh fence, which guided rabbits over a trap-door in the top of a box, which was buried in the ground. Prior to and during each trapping session, any holes in the fence were blocked to encourage the use of the traps. Chopped carrot was provided in the box traps, to mitigate lost grazing time, particularly for young juveniles. Smeuse traps consisted of wooden tunnels with doors that closed behind rabbits that entered. Box and smeuse traps were inserted on existing 'runs' so that rabbits would habituate to them. Cage traps were baited with chopped carrot and had a door whose restraining catch was released by a lever attached to a treadle, so that the door closed behind the rabbit as it entered. Five box traps were used at Exminster, eight at Frensham and five at Logiealmond. Seven smeuse traps were used at Exminster and Frensham, but they were not used at Logiealmond. Twenty cage traps were used on all three sites; at Exminster and Frensham they were used on all occasions, but they were only available at Logiealmond in May and July 1999. Trapping was performed at 6-week intervals and lasted for 3–5 consecutive days (2–4 nights). All traps were checked every 2–3 h during the daytime, from dawn until dusk. Cage and smeuse traps were not used overnight, whereas box traps caught rabbits predominantly overnight. Rabbits caught more than once in a single trapping session were released immediately.

All rabbits caught during each trapping session were weighed, examined and fitted with an individually numbered chick-wing tag (Ketchum Tags, Tadworth, Surrey, UK) in the ear, 10–15 mm above the occiput, unless they had been tagged previously. To prevent infection, Savlon antiseptic cream was applied to the tag before insertion. Each rabbit caught on each trapping session provided a small blood sample (up to $\sim$1 ml), which was taken from the marginal ear vein by shaving the region of the ear and cleaning it using a 'wet wipe' before making a small incision using a sterile blood lancet (BDH Ltd, Poole, Dorset, UK). Blood was collected in a 1·6 ml microcentrifuge tube and the flow then staunched. Savlon antiseptic cream was applied to the ear after blood sampling. In cold weather, rabbits were warmed using blankets and hot-water bottles, to encourage blood circulation through the ear. Following blood sampling, they were 'reacclimatized' to ambient temperature before release. Rabbits were released promptly at the site of their capture, after checking that they were in a suitable condition. The procedures performed in this study were covered by Home Office licences. To avoid the potential risk of spreading RHDV within and between the study populations, equipment and clothing were regularly disinfected using Virkon virucide (Antec International, Sudbury, Suffolk, UK).

Blood samples were kept cool until taken to the laboratory, where they were centrifuged twice at 10 000 ***g*** for 10 min to obtain serum. Sera were stored at $-20\ ^{\circ}$C prior to analysis. Details of ELISA-testing for antibodies against RHDV and RT–PCR testing for RHDV RNA are described in ref. [14]. The ELISA test was calibrated using 200 rabbit sera that had been independently assessed using the haemagglutination inhibition (HAI) test by the Veterinary Laboratories Agency (Weybridge, UK). The ELISA test results

correlated closely with those of the HAI test, indicating similar sensitivity and specificity. The ELISA test antigen does not react with sera from a wide range of animal species other than rabbit and some from hares (that tested positive by haemagglutination inhibition). Antibody titres were estimated by testing samples at final dilutions ranging from ten-fold to 1280-fold. Samples were regarded as positive if their optical density reading was twice that of the negative control at ten-fold final dilution.

To detect the presence of RHDV and to identify the strains that were present in the study populations, RT–PCR and nucleotide sequencing were performed, as described in ref. [14]. Serum samples from tagged rabbits were tested, and on each trapping occasion, the sites were searched thoroughly for cadavers, which were collected and analysed. To prevent cross-contamination of samples each cadaver was bagged separately and dissected in the laboratory under sterile conditions. To obtain tissue samples from healthy rabbits, in addition to serum samples, unmarked rabbits were shot at Logiealmond (10 in May 1999, 28 in June 2000 and 24 in June 2001).

The age of rabbits was estimated from their mass using site-specific growth curves, fitted by nonlinear least squares regression. Rabbits affected by myxomatosis were excluded from growth-curve fitting, since this disease causes loss of body condition. Four functions were tested: Gompertz, Logistic, von Bertalanffy and Richards [15], with von Bertalanffy performing best, with the lowest AIC (Akaike information criterion), lowest residual standard error, and the smallest median bias in the residuals. Within each site the effects of sex, year of birth, the duration between first capture and first recapture, and their interactions were not significant. Following Cowan [16], we assumed that rabbits emerged at 3 weeks of age, with a mass of 180 g. The maximum age that could be estimated from mass was 33 weeks; older rabbits were considered adult.

Transmission of RHDV was detected at the population level using seroprevalence estimates and age–seroprevalence relationships. Individual-level analysis allows more sensitive detection of the timing of RHDV spreading and also discrimination between spreading of pathogenic and non-pathogenic strains. The spreading of pathogenic strains of RHDV was detected from increased mortality amongst seronegatives compared with seropositives. Seroconversion events tend to indicate spreading of non-pathogenic strains of RHDV: the very high case-fatality rate of pathogenic RHDV infection means that there are very few survivors whose seroconversions could be detected, except amongst rabbits aged <2 months (which do not usually die of RHD [17, 18]).

At the population level, seroprevalence data were analysed by applying the concept of the 'minimum number known to be alive' measure of population size, which was valid in this instance since seropositive individuals aged >10 weeks remained seropositive upon recapture. Individuals aged >10 weeks whose serological status was the same on capture and recapture were 'counted' as having been alive and of known serological status during the periods between those captures. Since individuals aged <10 weeks may have been seropositive solely due to maternal antibodies [19], and may have lost those maternal antibodies and seroconverted subsequently – i.e. there was a chance that they might not have been seropositive continuously – they were recorded as having been of unknown serological status until recaptured aged >10 weeks. Individuals whose serological status changed between captures were counted as being of unknown status between captures. Therefore individuals that seroconverted were of 'unknown' status for at least one inter-trapping period. For some individuals the seroconversion event could not be allocated to a single inter-trapping period and, therefore, they were of unknown status for two or more inter-trapping periods. Thus for each trapping occasion, there were estimated the minimum numbers known to be seropositive (MNKTBSp), seronegative (MNKTBSn), and of unknown status (MNKTBU), whose sum equals the minimum number known to be alive (MNKTBA). Upper and lower estimates of seroprevalence were calculated as follows: lower estimate = MNKTBSp/MNKTBA; upper estimate = (MNKTBSp + MNKTBU)/MNKTBA.

At the individual level, the timing of seroconversion events was examined. Potential detection of the seroconversion of an individual rabbit required that it be caught whilst seronegative and subsequently recaptured. For each inter-trapping period, the maximum number of seroconversion events that could have been detected if they had occurred was calculated. This was the number of individuals that were seronegative when captured prior to that period and were recaptured after that period. Note that because individuals were not caught on every trapping session, in many cases it was not possible to determine the precise period when seroconversion would have occurred, but nevertheless that event would have

Table 1. *Summary of rabbit trapping results at the three study sites*

Site	Exminster	Frensham	Logiealmond
Period of trapping	March 1999–July 2001	March 1999–July 2001	May 1999–February 2001
No. rabbits tagged (female, male)	256 (119, 137)	164 (77, 87)	390 (216, 174)
No. tagged rabbits recaptured (female, male)	98 (48, 50)	62 (30, 32)	87 (47, 40)
Proportion of tagged rabbits recaptured* (female, male)	38 % (40 %, 36 %)	38 % (39 %, 37 %)	22 % (22 %, 23 %)
No. capture events	578	319	495
No. blood samples†	552	277	491 (trapped), 72 (shot)
Seroprevalence			
Mean (first captures)‡	74 % (175/236)	87 % (109/126)	95 % (367/386)
Range (first captures of each session)	59–100 %	73–100 %§	85–100 %
No. tagged cadavers found	3	2	8

* Note that these figures include those caught on the last trapping session, that had no opportunity to be recaptured: 21 at Exminster, 30 at Frensham and 6 at Logiealmond.

† Samples suitable for analysis were not obtained at every capture event.

‡ The denominators are less than the total number of rabbits that were tagged because samples suitable for analysis were not obtained from every rabbit upon first capture.

§ Excluding the anomalous result of 33 % in January 2000 when only three individuals were first-captured.

been detected. For each inter-trapping period, the minimum number of seroconversion events known to have occurred; the maximum number that may have occurred (note that several individuals are thus 'counted' several times); and the 'average' number of events likely to have occurred were calculated. This 'average' was estimated using a weighting system in which the 'likelihood' of an individual having seroconverted during a particular period was calculated as the reciprocal of the number of 'candidate' periods during which that seroconversion may have occurred. Details of the analysis of mortality rates are presented elsewhere [20].

RESULTS

At Exminster and Frensham, most of the population was tagged: the proportion of those rabbits caught on each trapping session that had already been tagged was typically approximately 90 %, except during the breeding season (results not shown). However at Logiealmond this proportion exceeded 50 % on only one occasion, despite more rabbits having been tagged than on the other sites (Table 1). Further, the proportion of tagged animals that was recaptured was significantly lower at Logiealmond that at the other two sites, which were not significantly different from each other (all sites: $\chi^2 = 318{\cdot}265$, 2 D.F., $P < 0{\cdot}001$; pairwise comparisons: Exminster–Frensham: $\chi^2 = 0{\cdot}010$, 1 D.F., $P > 0{\cdot}921$; Exminster–Logiealmond: $\chi^2 = 19{\cdot}296$, 1 D.F., $P < 0{\cdot}001$; Frensham–Logiealmond: $\chi^2 = 14{\cdot}102$, 1 D.F., $P < 0{\cdot}001$) (Table 1). The explanation for these results is that the Logiealmond population lived in a large warren complex on open moorland, and could not be surrounded completely by traps, unlike the other two populations, which lived in more restricted habitat.

Within each population, similar numbers of females and males were tagged, with there being a statistically significant difference only at Logiealmond, where 55 % of those caught were female ($\chi^2 = 4{\cdot}523$, 1 D.F., $P < 0{\cdot}034$). At the other two sites, more males were tagged than females, but the differences were not significant (Exminster: $\chi^2 = 1{\cdot}266$, 1 D.F., $P > 0{\cdot}260$; Frensham: $\chi^2 = 0{\cdot}610$, 1 D.F., $P > 0{\cdot}435$). There was no sex difference in the proportion of those tagged that were recaptured (Exminster: $\chi^2 = 0{\cdot}397$, 1 D.F., $P > 0{\cdot}528$; Frensham: $\chi^2 = 0{\cdot}083$, 1 D.F., $P > 0{\cdot}773$; Logiealmond: $\chi^2 = 0{\cdot}084$, 1 D.F., $P > 0{\cdot}771$; all sites combined: $\chi^2 = 0{\cdot}009$, 1 D.F., $P > 0{\cdot}922$).

Immune response to RHDV

All individuals found to be seropositive on a particular trapping session were always found to be seropositive when recaptured ($n = 225$: Exminster, 83;

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Frensham, 58; Logiealmond, 84), with the possible exception of a few young juveniles (see below). This applied both to those that were seropositive when first captured and those that seroconverted after their first capture. Thus actively acquired immunity appears to be life-long, at least in our study areas. Juveniles aged <10 weeks may have had maternal antibodies when first captured and thus may have lost their seropositivity before becoming exposed to RHDV and seroconverting. This may have occurred in up to four cases that were detected (see below). The minimum numbers of weeks that recaptured individuals were known to have remained seropositive were (range, mean, median): Exminster (6–114, 40·8, 30); Frensham (6–90, 30·6, 24); Logiealmond (6–84, 27, 24). There was no evidence for waning of antibody titres over time for those aged >10 weeks (results not shown).

Seroprevalence amongst individuals on their first capture was high at all three study sites (Table 1), ranging from 59 to 100% (excluding an anomalous result of 33% at Frensham in January 2000 when only three individuals were first-captured). There was a significant difference in mean seroprevalence amongst the first-captures on each site ($\chi^2 = 56{\cdot}856$, 2 D.F., $P < 0{\cdot}001$), with all three sites being significantly different from each other (pairwise comparisons: Exminster–Frensham: $\chi^2 = 7{\cdot}418$, 1 D.F., $P < 0{\cdot}007$; Exminster–Logiealmond: $\chi^2 = 57{\cdot}219$, 1 D.F., $P < 0{\cdot}001$; Frensham–Logiealmond: $\chi^2 = 10{\cdot}672$, 1 D.F., $P < 0{\cdot}002$). There was no significant difference in seroprevalence amongst rabbits that were shot at Logiealmond and those that were trapped. Seroprevalence amongst shot rabbits was as follows: May 1999: 90% (9/10); June 2000: 93% (26/28); June 2001: 92% (22/24).

Seroprevalence was high in all three study populations (Fig. 1). At Logiealmond it was consistently high (Fig. 1*c*). At Frensham there may have been a trend of increasing seroprevalence over the duration of the study, but it was slight (Fig. 1*b*). Interestingly, at Exminster seroprevalence remained approximately constant, for at least 48 weeks, until March 2000; then, at the time of emergence of the earliest-born of the year, it increased markedly – from 68 to 93% (the mid-point seroprevalence estimates of Fig. 1*a*) – in 12 weeks. Seroprevalence then remained high and approximately constant at least until the end of the study (i.e. at least 42 weeks); only 5 out of 88 rabbits (6%) first-caught from June 2000 at Exminster were seronegative.

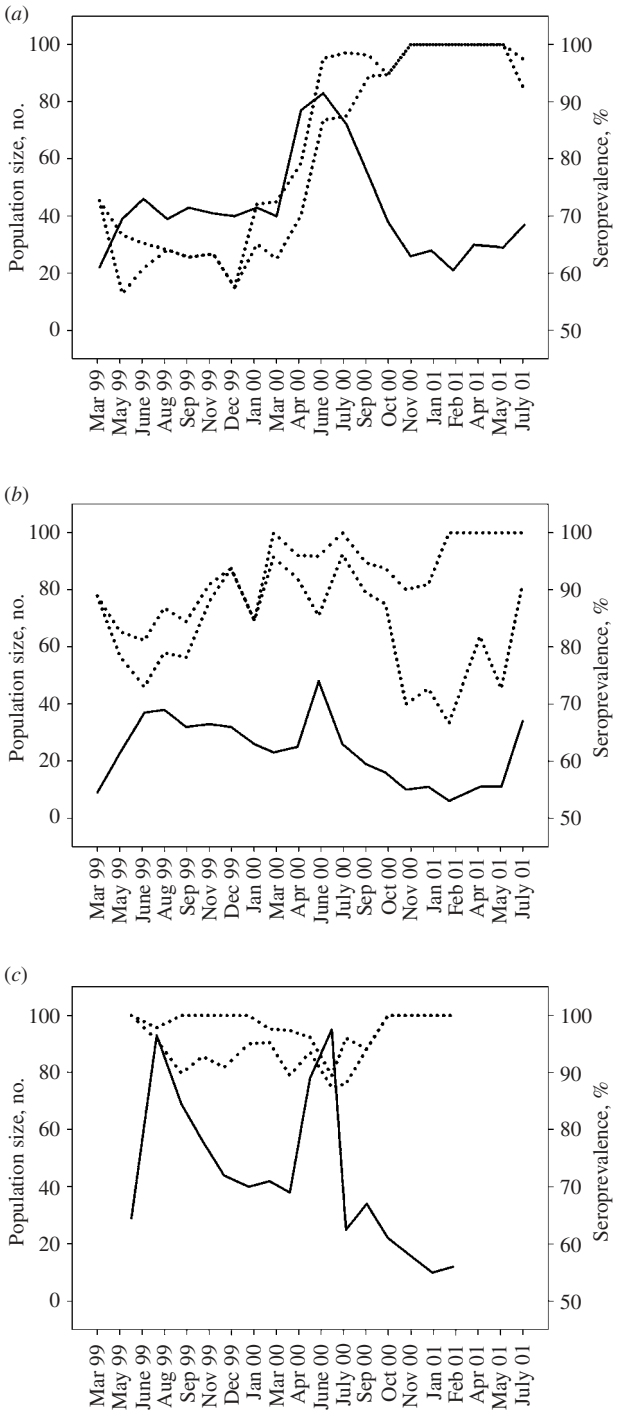

Fig. 1. Population size (primary axis) and seroprevalence (secondary axis) estimates for (*a*) Exminster, (*b*) Frensham, (*c*) Logiealmond. Upper and lower seroprevalence estimates were calculated using the concept of minimum numbers known to be seropositive, seronegative and of unknown serological status on each trapping occasion (see Methods for more details). Population sizes were the minimum numbers known to be alive. Note that population sizes towards the end of the study periods are underestimated by this method. —, Population size; ·····, seroprevalence.

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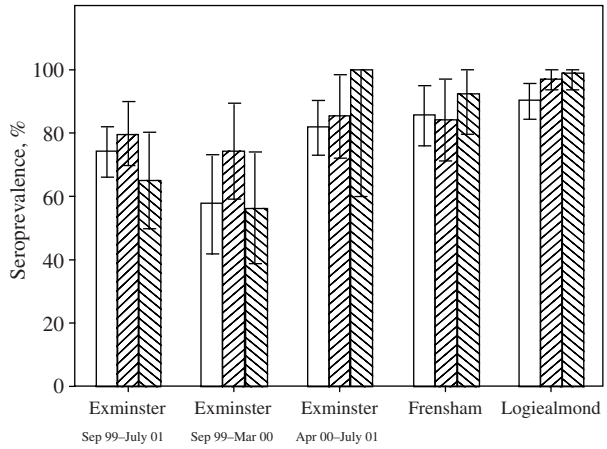

Fig. 2. Age-seroprevalence profile of rabbits from each study site. Individuals are only counted on their first capture. Data for Exminster are shown for the entire study period and also for the periods up to March 2000 and from April 2000. Error bars show 95% confidence intervals. □, <10 weeks; ▨, 10–33 weeks; ▧, >33 weeks.

Age–seroprevalence relationships

Seroprevalence was high in all age categories, in all three study populations, with a range of 56–100%, mean 84% (Fig. 2). For all sites, there were no sex differences in seroprevalence amongst any of the age categories, nor when the data were pooled across all age categories (Exminster: $\chi^2=0{\cdot}161$, 1 D.F., $P>0{\cdot}688$; Frensham: $\chi^2=0{\cdot}049$, 1 D.F., $P>0{\cdot}824$; Logiealmond: $\chi^2=0{\cdot}049$, 1 D.F., $P>0{\cdot}824$; all sites combined: $\chi^2=0{\cdot}147$, $P>0{\cdot}701$).

In light of the marked increase in seroprevalence at Exminster, primarily during March–June 2000 (Fig. 1), the age–seroprevalence data-set for that site was divided into those rabbits first-caught up to March 2000 and those first-caught afterwards, as well as being analysed in its entirety. At Exminster and Frensham there were no significant differences in seroprevalence amongst the different age categories [Exminster (March 1999–July 2001): $\chi^2=2{\cdot}861$, 2 D.F., $P>0{\cdot}239$; (March 1999–March 2000): $\chi^2=3{\cdot}037$, 2 D.F., $P>0{\cdot}218$; (April 2000–July 2001): $\chi^2=1{\cdot}902$, 2 D.F., $P>0{\cdot}386$; Frensham: $\chi^2=0{\cdot}977$, 2 D.F., $P>0{\cdot}613$]. At Logiealmond there was a significant – but relatively small – increase in seroprevalence from <10 weeks to the older age categories (Fig. 2) ($\chi^2=11{\cdot}374$, 2 D.F., $P<0{\cdot}004$; pairwise comparisons: <10 *vs.* 10–33 weeks: $\chi^2=5{\cdot}755$, 1 D.F., $P<0{\cdot}017$; <10 *vs.* >33 weeks: $\chi^2=7{\cdot}722$, 1 D.F., $P<0{\cdot}006$; 10–33 *vs.* >33 weeks: $\chi^2=1{\cdot}176$, 1 D.F., $P>0{\cdot}278$). These data imply that most infection occurred at an early age in all three populations.

Seropositive rabbits aged <10 weeks may have had maternal antibodies, but to be seropositive at older ages they must have been exposed to RHDV antigen and developed their own immune response. In most cases, that exposure seems to have occurred prior to (or very soon after) the loss of maternal antibodies, since of 64 seropositive rabbits that were first-caught when aged <10 weeks and then recaptured and tested on the following trapping session, 60 were seropositive (and remained so subsequently); two were seronegative upon recapture; and two others' titres had declined markedly and so may have been destined to 'lose' seropositivity also. Therefore, at most only 6% (4/64) may have 'lost' seropositivity and been recaptured; others may have lost their protection and then have died following RHDV infection. Furthermore, there was no significant difference in seroprevalence amongst rabbits first-captured aged <10 weeks and those first-captured aged 10–15 weeks [Exminster (March 1999–July 2001): $\chi^2=1{\cdot}560$, 1 D.F., $P>0{\cdot}211$; (March 1999–March 2000): $\chi^2=2{\cdot}770$, 1 D.F., $P>0{\cdot}096$; (April 2000–July 2001): $\chi^2=0{\cdot}030$, 1 D.F., $P>0{\cdot}862$; Frensham: $\chi^2=1{\cdot}271$, 1 D.F., $P>0{\cdot}260$; Logiealmond: $\chi^2=1{\cdot}668$, 1 D.F., $P>0{\cdot}196$].

Timing of seroconversion events

At Frensham and Logiealmond there was very little potential to detect seroconversions and only four and two seroconversions were detected respectively. However, note that all seroconversions detected at Frensham, and at least one – and maybe both – of those detected at Logiealmond, involved adults. Three of the four events at Frensham occurred during January–May 2000 (two of those during January–March); the other occurred during September 1999. At Logiealmond one event occurred during April–May 2000 (involving an adult); the other occurred during July 1999–May 2000.

At Exminster there was substantial potential to detect seroconversions during just over half of the study, and 17 seroconversion events were detected, of which 15 (including all detected 'adult' seroconversions) occurred during December 1999–October 2000 (Table 2). At least eight – and possibly all – of those 15 seroconversions occurred during a brief episode around March–June 2000. (Note that the 'average' measure probably underestimates the 'peak' incidence and overestimates the tails, given that seroconversion events are likely to be temporally 'clustered'.) Note that all individuals known to be seronegative

Table 2. *Timing of seroconversion events that were detected at Exminster*

Inter-trapping period beginning …	1999							2000						
	Mar	May	June	Aug	Sep	Nov	Dec	Jan	Mar	Apr	June	July	Sep	Oct
Inter-trapping period no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Max. no. seroconversions potentially detectable	5	7	10	11	13	15	14	14	11	11	6	4	3	0
No. rabbits *known* to have seroconverted (excluding juveniles)				1 (0)					4 (3)	4 (0)				
Max. no. rabbits that *may* have seroconverted (excluding juveniles)	1 (0)	1 (0)	1 (0)	1 (0)			3	4	11 (10)	10 (6)	5	3	1	
'Average' no. rabbits that seroconverted (excluding juveniles)	0·33 (0)	0·33 (0)	0·33 (0)	1 (0)			0·75	1	5·78 (4·78)	5·45 (1·45)	1·2	0·62	0·2	
Calculation of 'average' (for those whose actual period of seroconversion is not known)														
E712							0·33	0·33	0·33					
E721 (juvenile)	0·33	0·33	0·33											
E787									0·25	0·25	0·25	0·25		
E795									0·33	0·33	0·33			
E798							0·17	0·17	0·17	0·17	0·17	0·17		
E800							0·25	0·25	0·25	0·25				
E802									0·2	0·2	0·2	0·2	0·2	
E807								0·25	0·25	0·25	0·25			

Since the particular inter-trapping period in which the seroconversion event occurred is not known in all cases, three analyses are presented: the number of rabbits known to have seroconverted in each inter-trapping period; the maximum number that may have seroconverted in each period (which counts some individuals several times); and the 'average' estimate, which is explained in the Methods section. Numbers in parentheses indicate the values when juveniles were excluded from the analysis, in cases where doing so altered the results. No seroconversions could be detected after inter-trapping period 13. The lower part of the table shows the calculation of the 'average' estimates: e.g. rabbit E712 seroconverted during one of three periods (7, 8 or 9) and thus contributes a weight of 0·33 to each of those three periods; whilst E787 seroconverted during one of four periods and so contributes 0·25 to each of them.

when trapping was carried out in March 2000 were either never recaptured or had seroconverted by the time of recapture. All 16 seronegatives caught after March 2000 were recently emerged, first-captured juveniles. Of those, four were recaptured and tested, and all had seroconverted.

Prior to March 2000 little seroconversion activity had been detected at Exminster [and seroprevalence had remained roughly constant for 42 weeks (Fig. 1*a*)]. Spreading of non-pathogenic RHDV prior to December 1999 would probably have been detected, if it had occurred, since there existed the potential to detect seroconversions for at least 36 weeks prior to March 2000 (Table 2). Eight rabbits remained seronegative for a substantial period before seroconverting during (and perhaps just after) March–April 2000. The mean minimum age of individuals that seroconverted was 55 weeks (median 56 weeks) and the mean minimum time that those individuals had been known to have been present in the population prior to seroconversion was 29 weeks (median 27 weeks). (In fact it is likely that that they had been present in the population since emergence.)

In conclusion, amongst adults at Exminster, non-pathogenic RHDV appeared to have spread rapidly around March–June 2000, with little activity having occurred during the study prior to then. However, it is worth noting that RHDV spread to young juveniles at other times, as indicated by the age–seroprevalence data – but those data do not indicate whether the RHDV was pathogenic or

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non-pathogenic. After October 2000, spreading of non-pathogenic RHDV could not have been detected at Exminster since no individuals were caught whilst seronegative and subsequently recaptured (Table 2).

Spreading of pathogenic RHDV

An attempt was made to detect the spreading of pathogenic RHDV by its effect of increasing mortality of seronegatives (aged >2 months) compared to seropositives during periods when it was spreading. The findings are summarized here and reported in detail elsewhere [20]. In practice statistical power was lacking, due to the relatively small numbers of seronegatives and, in the case of juveniles, their high mortality rates irrespective of serological status. At Frensham and Logiealmond there was no evidence of periods of increased mortality of seronegatives of any age. However at Exminster there may have been increased mortality of seronegative adults around March–April 2000, when seronegative adults had an eight-fold higher risk of mortality than seropositive adults, although the number of tagged individuals apparently killed (3–6) was small [20]. When data were pooled over the duration of the study, there was no difference between the mortality rates of seronegative and seropositive adults, indicating that the risk of RHDV-induced mortality was transient.

It was striking that the time when the death rate of seronegative adults was significantly higher than seropositive adults was also the time when most (adult) seroconversion activity occurred (Table 2), which was the beginning of the breeding season: on the following trapping session, the first newly emergent young of the year were caught. Of nine seronegative adults caught in March 2000, three were never recaptured and all of the other six had seroconverted before subsequent recapture, which was by the following trapping session for half of them. Indeed, all seronegative individuals known to have survived March–April 2000 had seroconverted by their subsequent recapture. The apparent case-fatality rate of seronegative adults exposed to RHDV during March–April 2000 was estimated to be 25–30% [20].

Detection of RHDV strains

Although pathogenic RHDV did not appear to have been a substantial cause of mortality on any of the study sites, four rabbits were observed apparently succumbing to the disease in July 2000: three at Exminster and one at Logiealmond. In all cases the rabbits were in good condition, and died in the characteristic posture of RHD mortality, with the legs extended and the head tilted backwards. All subsequently tested positive for RHDV RNA by RT–PCR. In addition, RHDV RNA was detected in a number of other rabbit cadavers which were collected from the three study sites. (Most of those were partially decayed so it was not possible to determine their condition when they died, nor their posture.)

However, healthy rabbits from all three of the study sites were found to have persistent infection with RHDV: RT–PCR detected RHDV RNA in 8/21 (38%) seropositive serum samples that were taken from tagged rabbits that had seroconverted since their first capture, were healthy when sampled, and were subsequently recaptured. Serum samples obtained upon recapture were still found to contain detectable RHDV RNA. In addition, solid tissue samples (liver and, in some cases, bone marrow) from healthy rabbits that were shot at Logiealmond and elsewhere in the United Kingdom during the study period were found to contain RHDV RNA. As reported in ref. [14], some of the RNAs from serum and solid tissue samples were subjected to nucleotide sequencing and phylogenetic analysis of 527 nucleotides encoding part of the VP60 capsid protein. The surprising result was that the same branches of the phylogenetic 'tree' were occupied by RHDV RNAs in the following categories: (i) those detected in samples from healthy rabbits, including tagged rabbits known to have remained healthy; (ii) those detected in cadavers of apparent victims of RHD whose deaths were observed at Exminster and Logiealmond; and (iii) those belonging to virus isolates that had been found to be pathogenic by other workers [8, 21]. The 'Italian' non-pathogenic strain discovered by Capucci and colleagues [5–7] was not detected in any of our samples taken from healthy rabbits from the three study sites and elsewhere in the United Kingdom that were tested by RT–PCR and nucleotide sequencing.

These findings are consistent with those of a study of wild rabbits in New Zealand, which detected RHDV by RT–PCR in the livers of 38/76 (50%) healthy rabbits (of unknown serological status) that were shot, indicating persistent infection [22]. Again, phylogenetic analysis found no characteristic

differences between any of these strains and RHDV strains that other workers had found to be pathogenic. Another study [23] found persistent infection in the livers of 10/19 healthy wild rabbits in New Zealand, involving an RHDV strain that had 99% homology to known pathogenic strains.

DISCUSSION

This paper reports the first detailed study of the epidemiology of RHDV in UK wild rabbit populations. In the United Kingdom, high RHDV seroprevalence had been reported in wild populations in the absence of apparent mortality associated with RHD [10–12]. A putative non-pathogenic strain was proposed to be the explanation for this phenomenon. By longitudinal serum sampling of marked rabbits and collection of tissue samples from live rabbits and cadavers for analysis, this study addressed the following questions. Was a putative non-pathogenic strain of RHDV apparently endemic – i.e. did transmission (detected by seroconversions and/or an increase in seroprevalence) occur without associated mortality amongst seronegatives – and, if so, then when did that transmission occur? Were there any outbreaks of pathogenic RHDV and what was their impact on the population? How long did immunity to RHDV last and were there any signs of it waning?

This study did not find any evidence of waning immunity amongst repeatedly tested rabbits aged >10 weeks, which was consistent with other reports [5, 19]. The finding of RHDV RNA in serum samples from healthy rabbits suggests that the virus is able to persist in the infected host despite the presence of a strong immune response. Given the random selection of seropositive sera for testing by RT–PCR it is likely that virus persisted for a long time, in order to have been found so commonly. Although the absence of virus from some sera containing antibodies suggests that infection may be cleared eventually, this is not necessarily the case, for two reasons. First, even when RHDV was detectable its levels were very low, close to the limit of detection by the RT–PCR test, so failure to detect RHDV did not necessarily indicate its absence. Secondly, if latent infection had occurred then RHDV may not have been present in the serum when it was sampled, even though the rabbit was still infected in other tissue(s).

Seroprevalence was expected to exhibit a seasonal cycle, declining in the spring and summer due to the birth of naive juveniles, and increasing subsequently as the virus spread, as predicted by our seasonal, age-structured mathematical model [4]. Modification of the model to incorporate the acquisition of maternally derived antibodies reduced the amplitude of the predicted seasonal seroprevalence cycle, but did not abolish it [20]. It was expected that most rabbits aged <10 weeks would be seronegative and that most of those that were seropositive would have been so due to their having maternal antibodies, at relatively low titres. Seroprevalence was expected to increase with age, as individuals became exposed to RHDV and developed their own immunity, or died of infection. Contrary to expectations, no seasonal cycle of seroprevalence was apparent on any of the study sites, and seroprevalence did not increase substantially with age, indicating that some of the model assumptions may not have been valid. (Note that since it was not possible to determine whether antibodies against RHDV detected by the ELISA test were produced by the rabbit in response to exposure to pathogenic or non-pathogenic strains, this paragraph and the following two refer to 'RHDV' without specifying 'pathogenic' or 'non-pathogenic'. Remember that rabbits aged <2 months do not usually die following infection with 'pathogenic' RHDV [17, 18].)

The lack of a seasonal cycle in seroprevalence appeared to have been due to transmission of RHDV having occurred predominantly around the time of the breeding season, with little transmission having occurred at other times. In general, rabbits apparently experienced a high force of infection (per-susceptible rate of infection) during the first few weeks of life that did not continue as they aged. The evidence for this, from all three populations, was the high seroprevalence amongst <10-week-olds that was not substantially higher – or lower – in older age categories (Fig. 2). Although seropositive rabbits aged <10 weeks may have possessed maternally derived antibodies [19], most rabbits in all three populations must have been exposed to RHDV – and either survived to become immune or died of RHD – at young ages, since seroprevalence amongst those first-caught aged 10–15 weeks was not lower than that amongst those first-caught aged <10 weeks. Furthermore, detected 'loss' of seropositivity by rabbits first-caught aged <10 weeks that were recaptured on the following trapping session was rare.

At the same time of the year as young juveniles experienced a (transiently) high force of infection, so did adults (detected by seroconversions on all three sites, and higher mortality of seronegatives at

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Exminster). It is remarkable that the adults that seroconverted at Exminster remained seronegative for so long before seroconverting rapidly at the time of breeding in the year 2000. It is interesting to note that Cooke et al. [19] also found th at RHDV transmission may have been associated with breeding. The phenomenon may be due to at least two factors. First, the seasonal pattern of transmission could be due simply to the presence of susceptible individuals following breeding. Recently infected individuals are most likely to be infectious, so the increase in number of susceptibles – who then become infected – would increase the prevalence of infectious individuals in the population, thus increasing the force of infection. Secondly, in addition, it is possible that there is a seasonal cycle in the infectiousness of persistently infected rabbits. Given the high seroprevalence amongst rabbits too old to have maternal antibodies, on all three study sites, and the apparently long duration of infection (despite the presence of antibodies) in many rabbits, it is likely that, at any point in time, many of the rabbits in the populations were infected. The episodic transmission of RHDV, predominantly around the time of the breeding season, implies that despite rabbits probably being *infected* for a substantial period, they may only be *infectious* for part of that time. [Consistent with this, evidence from New Zealand found that RHDV was present in the livers of $\sim 50\%$ of healthy wild rabbits (detected by RT–PCR), but that its levels were very low [22, 23].] Perhaps RHDV causes latent infection, in which infected rabbits are episodically infectious – when viral titres would be raised – such that there are a few individuals infectious at any particular point in time. This may be modulated by a strong seasonal cycle, with many more individuals being infectious around the time of the breeding season than at other times, perhaps related to hormonal changes associated with breeding activity. A possible manifestation of this may be that transmission of RHDV occurred predominantly from mother to offspring during birth or subsequently (e.g. during suckling). Those recently infected juveniles may then have transmitted to other rabbits, including the adults whose seroconversions were detected.

RHDV-induced mortality

The only way to analyse the impact of RHDV on rabbit mortality in this study was through the comparison of mortality rates of seronegative and seropositive rabbits through time. Direct quantification of RHD deaths from analysis of cadavers was not possible because the length of the inter-trapping period gave plenty of opportunity for cadavers to decay or be scavenged before discovery, with the result that few were found in a good enough condition for examination. Furthermore, post-mortem examinations were not performed to identify the cause of death, and the detection of RHDV RNA in a cadaver did not necessarily indicate that RHD was the cause of death, since many healthy rabbits were found to carry RHDV RNA, and pathogenic strains could not be distinguished from non-pathogenic ones using nucleotide sequence data.

The apparent impact of RHDV serological status on adult mortality during the March–April intertrapping period at Exminster was intriguing, although care must be taken when considering a unique event that involved relatively few individuals. The apparent case-fatality rate of 25–30% experienced by seronegative adults exposed to RHDV during March–April 2000 was substantially less than the 95% expected for pathogenic strains of RHDV, but still substantially greater than the 0% expected for non-pathogenic RHDV. Perhaps both pathogenic and non-pathogenic strains of RHDV were spreading, although it was not possible to exclude the possibility that there was a single, distinct strain of RHDV of intermediate virulence with a case-fatality rate of only 25–30%. An intriguing possibility that may explain this apparent case-fatality rate is that a single strain of RHDV may have both 'pathogenic' and 'non-pathogenic' modes of behaviour, as discussed below.

In summary, it appeared that the spreading of RHDV occurred episodically. Age–seroprevalence data indicated that high rates of exposure to RHDV were experienced by juveniles <10 weeks old, but not usually by older individuals. Since pathogenic RHDV does not usually kill rabbits aged <2 months it was not possible to determine whether young juveniles were exposed to pathogenic or non-pathogenic RHDV. At Exminster during (and perhaps soon after) March–April 2000, adult rabbits appeared to experience high rates of exposure to both pathogenic and non-pathogenic RHDV. Interestingly, this was at the start of the breeding season.

Were there distinct pathogenic and non-pathogenic strains of RHDV in the study populations?

We expected to find non-pathogenic strains of RHDV (possibly the 'Italian' strain [5–7]) in some of the

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healthy seropositive rabbits, and possibly to find pathogenic strains in dead rabbits – if they had died of RHD. Intriguingly, despite the high seroprevalence in the absence of apparent mortality or depression of the populations due to RHD, despite the detected seroconversion events, and despite the detection of RHDV RNA in serum and solid tissue samples taken from healthy rabbits, no distinct non-pathogenic strain of RHDV was found [14]. Phylogenetic analysis found that RHDV RNAs detected in our serum samples, taken from healthy seropositive rabbits, occupied the same branches of the 'tree' as RNAs of pathogenic RHDV strains that were reported by other workers [8, 21]. Thus no apparent characteristic differences were identified in the region of the genome tested. In addition, we found that serum samples collected from captive rabbits in the periods 1955–1961 and 1971–1980 contained detectable RHDV in the presence of antibodies against the virus in 5/10 cases and 6/10 cases respectively, and again phylogenetic analysis did not find any characteristic differences from the isolates known to be pathogenic [14]. Two studies [22, 23] found that strains of RHDV carried by healthy wild rabbits in New Zealand also could not be distinguished phylogenetically from 'pathogenic' strains. With respect to the current study, we suggest the following hypotheses:

(H_1) healthy rabbits were infected with a pathogenic strain of RHDV but infection occurred when they were aged <2 months old and so they did not die [17, 18];

(H_2) the same strain of RHDV has the capacity to propagate itself through both pathogenic and non-pathogenic modes of behaviour;

(H_3) the strain of RHDV responsible for the immunity-without-mortality *was* a distinct non-pathogenic strain (different from the 'Italian' non-pathogenic strain), but it differed from pathogenic isolates of RHDV in a region of the genome that was not analysed.

Consistent with (H_1), it was clear (from the age–seroprevalence relationships for all three study sites) that most individuals in all three study populations were exposed at a very young age, in most cases probably before the age of 10 weeks, when maternally derived antibodies would have been lost (see above). However, since seroconversions of rabbits substantially older than 2 months were detected at Exminster and Frensham (and possibly also at Logiealmond, since one of the two rabbits that seroconverted did so during a 10-month period in between captures), (H_1) cannot be a complete explanation.

A plausible scenario consistent with hypothesis (H_2) was examined elsewhere by mathematical modelling [24]. We postulated that the outcome of infection with RHDV depends upon the size of the inoculum, with 'large' doses of virus leading to 'acute' pathogenic infection which transmits 'large' doses of virus that cause acute infection in others; whilst 'small' doses cause 'chronic' infection which transmits 'small' doses that cause chronic infection in others. Survivors of acute infection would develop chronic infection.

Laboratory studies are required to distinguish between hypotheses (H_2) and (H_3) – i.e. that a single strain of RHDV may exhibit both pathogenic and non-pathogenic modes of behaviour [(H_2)], or that there are distinct pathogenic and non-pathogenic strains present in the United Kingdom, and that the predominant non-pathogenic strain is different from the 'Italian' non-pathogenic strain [(H_3)]. Our phylogenetic analysis [14] used the region of the RHDV genome that has been most studied by other workers, in order to compare our nucleotide sequences to theirs, and also because the 'Italian' non-pathogenic strain can be identified from this region [5]. However determinants of virulence have not been identified and it is possible that they are located elsewhere in the genome. (Indeed it may be the case that the genomic differences identified for the 'Italian' non-pathogenic strain are *unrelated* to its being non-pathogenic.) Hence there may be other non-pathogenic strains of RHDV that differ from pathogenic strains in another region of the genome. To determine if the RHDV detected in healthy rabbits in this study is a distinct non-pathogenic strain or not, we are currently sequencing the whole genomes of the viruses sampled.

In summary the epidemiology of RHDV infection of rabbits may be more complex than previously realized. Specifically, the findings reported in this paper and elsewhere [14, 22, 23] are evidence for persistent infection that may be episodically infectious, with transmission apparently occurring predominantly around the time of the breeding season, and also that the same strain of RHDV may exhibit both virulent and avirulent modes of behaviour. In other words, 'pathogenic' and 'non-pathogenic' RHDV may be modes of behaviour exhibited by the same virus, rather than different strains. If this is the case

then the epidemiology of RHDV in the United Kingdom and Europe may be explained by a simple mathematical model [24]. The implications for the origins of RHDV and its emergence are discussed in ref. [20]. Further laboratory studies are required to clarify the situation, in addition to further mathematical modelling to examine the implications of the hypotheses proposed in this paper. Such studies are likely to make important contributions to our understanding of emerging diseases in general.

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